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Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

SUBSTANCES FROM ALFALFA BIOLOGICALLY ACTIVE AGAINST THE SPOTTED ALFALFA APHID [1/]

By A. N. Kishaba and G. R. Manglitz [2/] [3/]

During an investigation into the chemical nature of resistance in alfalfa to the spotted alfalfa aphid, Therioaphis maculata (Buckton), substances were extracted from alfalfa that elicited responses from this aphid. On the basis of these responses, the substances were classified as arrestants, deterrents, and toxins. The terms "arrestant" and"deterrent", as used here, correspond to usage in the literature.[4/] This paper reports results obtained with substances extracted from alfalfa that caused biological activity in the aphid.

MATERIALS AND METHODS

The susceptible alfalfa used included the varieties Caliverde, Chilean, and Dupuit; the resistant alfalfa used included clones C-84, C-89, and C-32 and selections from the varieties Lahonton and Moapa. When the extractions were made, all plants were either at prebloom or bloom stage. The extracts from which arrestant substances were obtained were made by grinding fresh trifoliolates from the alfalfa plants and then extracting with acetone, methanol, ethanol, or hexane, or any combination of these. Then the residue was dissolved in alkaline water (pH 9, $NaHCO_3$), the solution was extracted three times with anhydrous diethyl ether, and the ether phase was extracted three times with acidic water (pH 3, H_2SO_4) and then evaporated to dryness.

1/ Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, in cooperation with the Nebraska Agricultural Experiment Station. Published with the approval of the Director as Paper No. 2204, Journal Series, Nebraska Agricultural Experiment Station, Contribution No. 290, Department of Entomology, University of Nebraska.

2/ Formerly Department of Entomology, University of Nebraska, and Entomology Research Division, ARS, USDA, Lincoln, Nebr., respectively.

3/ The authors thank the following staff members of the University of Nebraska: R. Staples, R. E. Hill, H. J. Ball, and F. A. Haskins and the following ARS personnel: W. L. Howe, H. J. Gorz, and W. R. Kehr (who also supplied the plant material used) for their advice throughout the study.

4/ Dethier, V. G., Browne, B., and Smith, C. M. The designation of chemicals in terms of the response they elicit from insects. Jour. Econ. Ent. 53: 134-136. 1960.

This residue was dissolved with a small volume of diethyl ether, centrifuged to remove any insoluble residue, evaporated to dryness again, and then dissolved with acetone or methanol so that 1 ml. represented 1 g. of fresh alfalfa trifoliolates.

The extracts from which the toxic substances were obtained were prepared by soaking the aerial portions of plants that were in one-quarter bloom in benzene-ethanol (1 : 1, v./v.) at the rate of 4 ml. solvent per 1 g. plant material. This extract was filtered through glass wool and flash evaporated at 50° C. The residue was saponified with 1.2 N KOH (1 ml. per 1 g. of fresh weight) for 2 hours at 50° C. and then extracted four times with diethyl ether. The ether extract was washed with distilled water, dried with Na_2SO_4, and then concentrated to dryness. The dry residue was extracted with a mixture of diethyl ether-methanol (50-50, v./v.) and centrifuged, and this extract was finally concentrated, or diluted, or both to the volume equivalent of the weight of the starting material.

The extracts from which deterrent substances were obtained were prepared by soaking aerial portions of the plants in benzene-ethanol. This extract was then flash evaporated to dryness, dissolved with 65 percent ethanol, and passed through the cation exchange column (Dowex 50W-X8, 2 ml. per 10 g. of fresh material) modified after Mattocks. 5/ A flow diagram (fig. 1) presents the remaining details of this extraction process.

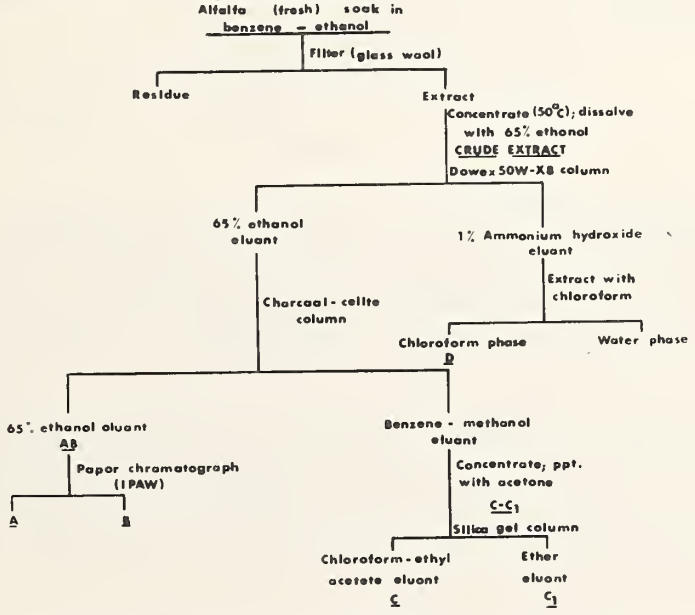

Figure 1. Flow diagram for the fractionation of the alfalfa indicating that several components are involved in the deterrent complex.

5/ Mattocks, A. R. Extraction of heat-labile alkaloids from plants. Nature 191: 1281-1282. 1961.

A bioassay was developed when preliminary tests showed that filter paper treated with hexane extract of leaves from C-84 and Caliverde elicited more response (number of aphids with their proboscises placed against the paper in a feeding position) than filter paper treated with distilled water alone. The checking for aphids in the feeding position was facilitated by confining the aphids in plastic cages that were 2.8 X 7.5 X 3.3 cm. Chromatographic paper (Whatman No. 1) was cut to size to line the bottom of the cages and was treated by dipping. The concentration was varied by the number of dips (interspersed by forced air drying). Both treated and untreated (check) filter papers were then moistened with a few drops of distilled water before 15 adult aphids were introduced into each cage. Each treatment was replicated six times. Observations were made hourly for 5 hours on the number of aphids in a feeding position. This type of bioassay was used to test arrestant and toxic substances.

A different method was used to assay for deterrent substances. Leaflets from the susceptible alfalfa plants were treated by dipping in the previously prepared plant extract (65 percent methanol solution) and arranged as previously described. 6/ Check leaflets were dipped in 65 percent methanol. Then 100 aphids were given a choice of 10 leaves (five treatments, two leaves each) in a single container (11.5 X 11.5 X 3.5 cm.). Each test was replicated five times. Five hours after infestation, the number of aphids feeding on each leaflet was recorded. The degree of activity of the test fractions was measured by comparing the number of aphids feeding on the treated leaflets with the numbers feeding on the check leaflets.

RESULTS AND DISCUSSION

Arrestant Substances

A typical response obtained from the arrestant fraction is shown in table 1. Similar methods were used to obtain active fractions from various alfalfa varieties and clones, resistant and susceptible, in nearly equal concentration. Further study showed that the active fractions could be obtained by soaking leaves in acetone, or methanol, or both without previous grinding. Also, aphid response could be obtained after a second extraction with methanol when the majority of the water soluble material was removed by first extracting with hot water (75° C.). The active components(s) was found to be saponifiable (1 M alkaline ethanol), and part of the activity was retained when the acidic and neutral fractions were combined. Some loss in activity occurred when the active components were adsorbed on charcoal and subsequently eluted with benzene.

6/ Kishaba, A. N., and Manglitz, G. R. Non-preference as a mechanism of sweetclover and alfalfa resistance to the sweetclover aphid and the spotted alfalfa aphid. Jour. Econ. Ent. 58: 566-569. 1965.

Table 1.--Typical arrestant response elicited from spotted alfalfa aphids confined in cage lined with moist filter paper treated with extract from the alfalfa plant

Relative concentration	Average number of aphids in feeding position 1/	
	Test 1	Test 2
0.01	--	2.2
.025	--	2.3
.05	--	5.2
.1	--	4.0
.25	8.2**	5.5*
.50	9.3**	6.5**
.75	7.2**	8.8**
1.0	9.8**	8.2**
2.0	5.3	7.8**
3.0	3.8	8.5**
4.0	3.8	--
.0	3.5	2.8
LSD at 5-percent level	2.7	2.5
LSD at 1-percent level	3.5	3.3

1/ Average from six replicates with 15 insects per replicate.
** Significant at 1-percent level of confidence.
* Significantly different from control (0.0) at 5-percent level of confidence.

Some unexplained difficulties were encountered. First, on some days, the number of aphids in feeding position on the check was high without a proportionate increase of feeding on the treated paper. Second, further purification gave erratic results. Even if activity occurred on the first trial, complete inactivity occurred after purification. Thus the entity with the arrestant activity may not be a specific material but a complex of materials or a few materials with additive or synergistic effects.

Arrestant substances in the host plants of other insects have been demonstrated. An example is cotton and the boll weevil. 7/ Arrestant extracts from cotton have elicited strong feeding responses in the boll

7/ Maxwell, F. G., Jenkins, J. N., Keller, J. C., and Parrott, W. L. An arrestant and feeding stimulant for the boll weevil in water extracts of cotton-plant parts. Jour. Econ. Ent. 56: 449-454. 1963.

weevil to green bean pods and other normally unattractive material. Unfortunately with a haustellate insect such as the spotted alfalfa aphid (feeding cannot be easily demonstrated) such dramatic results cannot be obtained. Furthermore, aphids may attempt to feed on any surface where they find themselves; thus the arrestant causes a continuation of feeding rather than initiating it.

The results of this preliminary study indicated that the arrestant was present in nearly equal concentration in both resistant and susceptible alfalfa plants.

Toxic Substances

In the bioassay for the toxin, the air-dried, treated paper was moistened as noted, with a few drops of buffer solution at pH 7 because the toxin was unstable in acidic solutions. The activity of each fraction was determined by making serial dilutions and noting mortality after a 5-hour exposure; each treatment was replicated six times with 15 aphids per cage. The LC_{50} was then approximated by plotting the data on log-probit paper. A typical result obtained with resistant plant material is shown in figure 2.

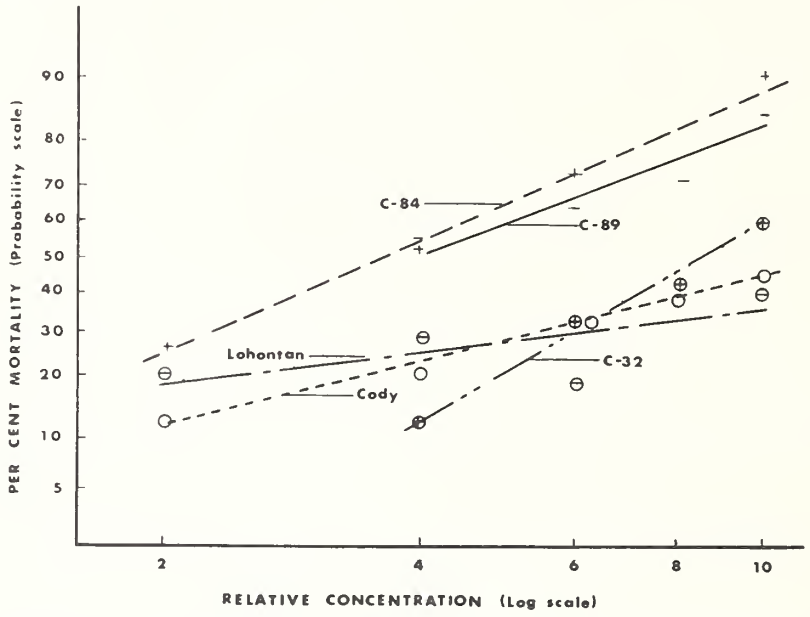

Figure 2. Dosage-response curves of the toxic fraction extracted from resistant alfalfa clones and/or varieties.

Numerous tests demonstrated that susceptible varieties had low toxicity. However, further testing throughout the year showed that the LC_{50}'s for extracts of susceptible material varied from 6 to 14 times the concentration of the starting material (fig. 3). Fractions from the resistant selections from Moapa and Lahonton possessed from 2 to 8 times more activity than the susceptible selections from the same varieties. Though these varieties are resistant, individual susceptible plants can be found within them. Differences of similar magnitude were found when extracts from the resistant clone C-84 and a susceptible clone, variety Caliverde, were compared.

The toxic component(s) has been found to be heat unstable and acid unstable and apparently required alkali treatment for activity.

During limited testing with the other assay in which leaves of Caliverde and Dupuit varieties were used as the treatment base, and extracts were applied to detached leaves of susceptible plants, a pronounced deterrent response was elicited without alkali treatment of the fraction.

The higher concentration of toxic substance(s) found in resistant alfalfa make it suspected of being the resistance factor. The great variation in results may merely reflect crude assay methods or indicate a need to refine the extraction process. However, in the plant, the resistance factor apparently does not act as a toxin. 8/ The toxic nature of the extracts may be an artifact produced by the alkali treatment as evidenced by the results when this extract, untreated with alkali, was applied to whole leaves.

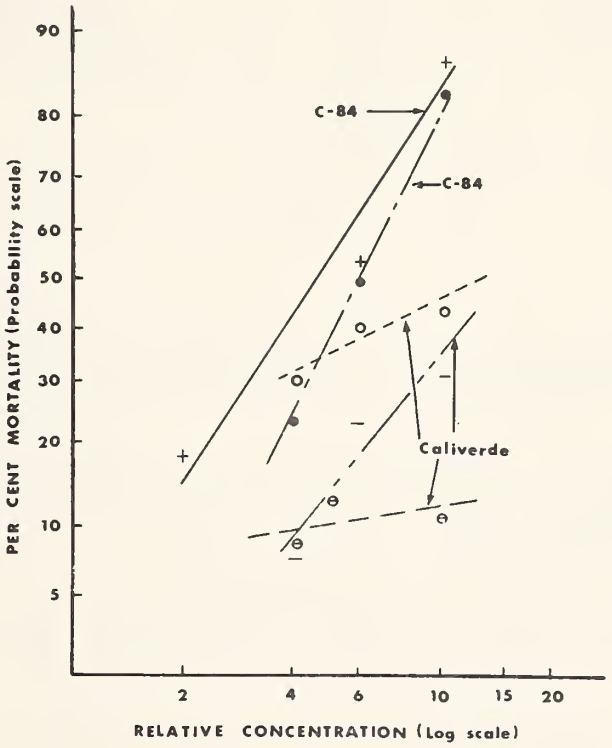

Figure 3. Dosage-response curves showing the variability of the toxic fraction extracted from resistant alfalfa clone C-84 and susceptible clonal selection from variety Caliverde.

8/ See footnote 6, p. 4.

Deterrent Substances

Because many active fractions were obtained from the deterrent studies, the relationship of these fractions to the process of extraction is best seen by use of a diagram (fig. 1). The crude extract was unstable at room temperature when dry, but it was stable in solution when it was stored in a freezer. A typical deterrent response, using treated leaflets, is shown in table 2.

Table 2.--Deterrent response elicited from spotted alfalfa aphids given a choice of susceptible alfalfa leaflets treated with alfalfa extract at various concentrations

Treatment: relative concentration of factors 1/		Mean number of aphids in feeding position at 5 hours 2/
AB	$C\text{-}C_1$	
0.25 X + 1 X		16.0
.5 X + .75 X		9.0
.75 X + .5 X		3.8
1 X + .25 X		2.4
Check (65 percent methanol)		24.2
LSD at 5-percent level		6.8
LSD at 1-percent level		9.4

1/ Concentrations are relative to wet weight of the plant sample (1.0 extract from 1 g. of plant sample diluted to 1 ml.)

2/ Mean number of aphids is based on a test replicated five times with 100 aphids per replicate.

The fractions designated C - C_1 obtained from preliminary silica gel fractionation, may be a complex (table 3); activity was not equivalent to the crude fraction (AB + C-C_1) until three fractions from the column were recombined (ether, chloroform, and ethyl acetate fraction). Complete elution of each eluant was checked by the gravimetric method. Paper chromatographic fractionation with the isopropanol-pyridine-water-acetic acid (8:8:4:1v./v./v./v.) solvent system 9/10/ showed that fractions AB also contained at least two components (table 4). Fraction D elicited a response opposite to that of the deterrent complex two out of three times (table 5).

The respective fractions from resistant and susceptible alfalfa were interchanged and assayed to determine whether fractions AB and C-C_1 were specific as to source. The optimum concentration of AB and the near minimum concentration of C-C_1 were used. No difference was noted (table 6).

The response of the aphid to whole plants or to detached plant parts indicates that resistance is caused by a material that deters feeding. However, in our studies, these substances appeared to be present in equal quantities in resistant and susceptible plants and (table 6) appeared to be identical in nature. Thus the deterrent compounds we studied may not be those responsible for resistance to the spotted alfalfa aphid in alfalfa. However, no definite conclusion should be made until isolation is achieved and a quantitative bioassay is developed.

9/ Gordon, H. T., Thornburg, W. W., and Wernum, L. N. Rapid paper chromatographic fractionation of complex mixtures of water-soluble substances. Jour. Chromatog. 9:44-59. 1962.

10/ Gordon, H. T., Thornburg, W. W., and Wernum, L. N. Rapid paper chromatography of carbohydrates and related compounds. Anal. Chem. 28:849-855. 1956.

Table 3.--Fractions from silica gel chromatogram showing that more than one factor may be involved in the benzene-methanol eluant from charcoal (C-C_1)

Treatment fractions	Mean number of aphids on leaflets 5 hours after infestation 1/
Ethyl acetate + chloroform + AB	9.8
Chloroform + ether + AB	10.4
Ether + benzene + AB	10.6
Benzene + hexane + AB	17.4
Check (65 percent methanol)	32.2
LSD at 5-percent level	7.1
LSD at 1-percent level	9.8
Ethyl acetate + chloroform + ether + AB	5.4
AB	18.0
AB + C-C_1	4.0
Check (65 percent methanol)	26.8
LSD at 5-percent level	7.8
LSD at 1-percent level	10.8

1/ Mean number of aphids is based on a test replicated five times with 100 aphids per replicate.

Table 4.--Fractions A and B from isopropanol-pyridine-acetic acid-water paper chromatogram of AB showing that more than one factor may be involved in AB

Treatment fractions	Mean number of aphids present on leaflets 5 hours after infestation 1/
A 2/ + $C\text{-}C_1$	17.0
A + B + $C\text{-}C_1$	5.4
AB (1 cc.) C-C	11.4
AB (2 cc.) $C\text{-}C_1$	3.0
Check (65 percent methanol)	25.0
B 2/ + $C\text{-}C_1$	12.0
Check (65 percent methanol)	30.4

1/ Mean number of aphids is based on a test replicated five times with 100 aphids per replicate.

2/ The concentration of fractions A and B eluted from the paper chromatogram was equivalent to 2 cc. of fraction AB.

Table 5.--Arrestant activity obtained with fraction D (from Dowex 50W-X8 column)

Treatment fraction	Number of aphids present on leaflets 5 hours after infestation 1/		
Chloroform phase D	28.2	42.6	36.6
Check (65 percent methanol)	26.8	22.0	26.4
LSD at 5-percent level	7.8	6.7	6.0
LSD at 1-percent level	10.8	9.2	8.0

1/ Mean number of aphids is based on a test replicated five times with 100 aphids per replicate.

Table 6.--Activity of the deterrent complex when factors AB and C-C_1 were interchanged with those from susceptible and resistant alfalfa clones by using the near minimum concentration of C-C_1 and the near optimum concentration of AB

Clone	Relative concentration		Relative		Number of aphids present on leaflets 5 hours after infestation 1/
	AB		C-C_1		
			Resistant	Susceptible	
C-89	1.0		0.125	---	3.0
Caliverde	1.0		.125	---	2.0
C-89	1.0		---	0.125	1.8
Caliverde	1.0		---	.125	1.8
Check (65 percent methanol)	---		---	---	52.0
LSD at 5-percent level					4.6
LSD at 1-percent level					6.4

1/ Mean number of aphids is based on a test replicated five times with 100 aphids per replicate.

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